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(54) Title: ANTIGEN COMPOSITION AGAINST MYCOPLASMA

10	20	30	40	50
1234567890	1234567890	1234567890	1234567890	1234567890
MKKMLRKFL	YSSAIYATSL	ASIIAFVAAG	CGQTESGSTS	DSKPQAETLK
HKVSNDSIRI	ALTDPDNPRW	ISAQKDIISY	VDETEAATST	ITKNQDAQNN
WLQQANLSP	APKGFIIAPE	NGSGVGTAVN	TIADKGIPIV	AYDRLITGSD
KYDWYVSDN	EKGELQGLS	LAAGLLGKD	GAFDSIDQMN	EYLKSHMPQE
TISFYTIAGS	QDDNNSQYFY	NGAMKVLKEL	MKNSQNKIID	LSPEGENAVY
VPGWNYGTAG	ORIQSFLTIN	KDPAGGNKIK	AVGSKPASIF	KGFLAPNDGM
AEQAITKLKL	EGFDTQKIFV	TRQDYNDKAK	TFIKDGDQNM	TIYKPDVKLG
KVAVEVLRVL	IAKKNKASRS	EVENELKAKL	PNISFKYDNQ	TYKVQGKNIN
TILVSPVIVT	KANVDNPDA			419

(57) Abstract

The present invention relates to a putative protective antigen against a *Mycoplasma*, prepared by a method including a sample of a *Mycoplasma*; an antibody probe including at least one antibody against a *Mycoplasma* produced by a method including: providing a biological sample taken a short time after an immune animal has been challenged with a *Mycoplasma* or *Mycoplasma* extract taken from the infection site or an area of a lesion or an area close to the infection site or lesion; isolating cells from the biological sample; culturing cells *in vitro* in a suitable culture medium; and harvesting antibodies produced from said cells; probing the *Mycoplasma* sample with the antibody probe to detect at least one antigen; and isolating the antigen detected, also including diagnostic antigens, the preparation thereof, and their use in the formation of vaccine compositions, particularly vaccine compositions against *Mycoplasma hyopneumoniae* infections.

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ANTIGEN COMPOSITION AGAINST MYCOPLASMA

The present invention relates to protective and diagnostic antigens, the preparation thereof, and their use in the formation of vaccine compositions, particularly vaccine compositions against Mycoplasma hyopneumoniae infections.

5 Mycoplasma hyopneumoniae is a ubiquitous swine respiratory pathogen causing mycoplasmal pneumoniae in swine (swine enzootic pneumonia). Swine enzootic pneumonia is probably the most widespread and economically significant disease in swine producing countries of the world. The economic effects of swine enzootic pneumonia (SEP) are complex, and the cost of the
10 disease is severe. In Australia, the disease was estimated in 1988 to cost approximately \$20,000,000 per annum. Increased mortality, decreased growth weight, depressed feed conversion, susceptibility to secondary bacterial infections, increased management costs, and increased use of antibiotics, are the main reasons for the economic impact of SEP.

15 Whilst several experimental vaccines have been produced, these have resulted in less than optimal results, and utilising various classes of antibiotics such as tetracycline, lincamycin and tiamulin is still the most widespread control treatment. Such antibiotics are, however, of limited therapeutic value, because they do not prevent the establishment of an infection, and lung lesions may
20 develop after treatment ends.

European Patent Application 359,919 to ML Technology Ventures L.P. describes a series of antigens, 36 kD, 41 kD, 74.5 kD and 96 kD in size, and proposes the use of such antigens in vaccines. Results presented suggest that some protection in pigs against challenge was achieved.

25 However, there remains a need in the art for an effective vaccine against M. hyopneumoniae which would confer protection against colonisation and clinical disease following M. hyopneumoniae challenge and also significantly reduce the morbidity and mortality from secondary infections.

Accordingly, it is an object of the present invention to overcome, or at least
30 alleviate, one or more of the difficulties and deficiencies in the prior art.

Accordingly, in a first aspect of the present invention there is provided a putative protective antigen against a Mycoplasma, preferably Mycoplasma

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hyopneumoniae prepared by a method including

providing

a sample of a Mycoplasma;

an antibody probe including at least one antibody against a

5 Mycoplasma produced by a method including;

providing a biological sample taken a short time after an immune animal has been challenged with a Mycoplasma or Mycoplasma extract taken from the infection site or an area of a lesion or an area close to the infection site or lesion;

10 isolating cells from the biological sample;

culturing cells in vitro in a suitable culture medium; and

harvesting antibodies produced from said cells;

probing the Mycoplasma sample with the antibody probe to detect at least one antigen; and

15 isolating the antigen detected.

The protective antigens may also function as diagnostic antigens as discussed below.

Accordingly, in a preferred aspect of the present invention there is provided a putative protective antigen against Mycoplasma hyopneumoniae, or related

20 infections, selected from the group of antigens having approximate molecular weights of 110-114, 90-94, 72-75, 60-64, 52-54 and 46-48 kilodaltons (kD), as hereinafter described, mutants, derivatives and fragments thereof. The putative protective antigen may be a surface protein. The putative protective antigen may be a surface lipoprotein or membrane protein.

25 Preferably the protective antigens are selected from the group of antigens having approximate molecular weights of 110-114, 90-94, 74, 62, 52 and 48 kD.

Preferably, the 72-75 kD antigen includes the following N-terminal amino acid sequence:

AGXLQKNSLLEEVWYAL

30 and, optionally, one or more of the following internal amino acid sequences:

AKNFDFAPSIQGYKKIAHEL

NLKPEQILQLLG

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LLKAEXNKXIEEINTXLDN

Preferably, the 60-64 kD antigen includes one of the following N-terminal amino acid sequences:

MKLA~~K~~LLKGFX(N/L)(M/V)IK

5 ADP(F/I)(R/E)Y(V/A)PQG(Q/A)X(M/N)VG

Preferably, the 52-54 kD antigen includes the following N-terminal amino acid sequence:

AGXWAKETTKEEKS

and, optionally, one or more of the following internal amino acid sequences:

10 AWVTADGTVN

AIVTADGTVNDNKPNQWVRKY.

Preferably, the 46-48 kD antigen includes the following N-terminal amino acid sequence:

AGXGQTESGSTSDSKPQAETLKHKV

15 and, optionally, one or more of the following internal amino acid sequences:

TIYKPDKVLGKVAEVLRVLIKKNKASR

AEQAITKLKLEGFDTQ

KNSQNKIIDLSPPEG

The 46-48 kD antigen may be encoded by a nucleic acid fragment:

20

	10 1234567890	20 1234567890	30 1234567890	40 1234567890	50 1234567890	
25	ATGAAAAAAA	TGCCACTATA	CCAGAGGAAA	GAGCAGTATA	TA AA ATAATT	50
	AAAATTACAT	TTTCTTCATT	TGCGCCAGAA	TTTTAAGAA	TTAGTACATT	100
	AAAAAGTAGA	ACAAAAGTTA	TTAATGTAAA	CATTAGCGCA	ATCCTTAAGA	150
	AAAAATTAAA	AGTTTTATCT	ATTTTTTTA	ATCGAAATCC	AACCAGGCAT	200
	AAATCTTGT	CAGTATTAT	CAAGTCGGTA	TTTTTCATT	ATTCTACTA	250
30	AAATATTATT	TGAATTTGCA	TTTTCATCAA	TCTAAAATT	TACATTTTTT	300
	TATAACAATT	TTTAAAAATT	ACTCTTTAAT	TTATAGTATT	TTTTTATTTT	350
	TTAGTCTAAA	TTATAAAATT	ATCTTGAATT	TTATTTGAAT	TTTTATAATT	400
	TAGTACTAAA	AAATACAAAT	ATTTTTCCCT	ATTCTAAGAA	AAATTCTATT	450
	TTTAAAAAAA	ATTGATTTT	ATAGTATAAT	TTGTTTGTAT	AATTGAATTA	500
35	ACTTGATTTG	AAAGGGAAACA	AAATGAAAAA	AATGCTTAGA	AAAAAATTCT	550
	TGTATTCATC	AGCTATTAT	GCAACTTCGC	TTGCATCAAT	TATTGCATT	600
	GTTGCAGCAG	GTTGTGGACA	GACAGAACATCA	GGTCAACTT	CTGATTCTAA	650
	ACCACAAGCC	GAGACGCTAA	AACATAAAGT	AAGTAATGAT	TCTATTGAA	700
	TAGCACTAAC	CGATCCGGAT	AATCCTCGAT	GAATTAGTGC	CCAAAAAGAT	750
40	ATTATTTCTT	ATGTTGATGA	AACAGAGGCA	GCAACTTCAA	CAATTACAAA	800
	AAACCAGGAT	GCACAAAATA	ACTGACTCAC	TCAGCAAGCT	AATTAAAGCC	850
	CAGCGCCAAA	AGGATTATT	ATTGCCCTG	AAAATGGAAG	TGGAGTTGGA	900

	ACTGCTGTTA	ATACAATTGC	TGATAAAAGGA	ATTCCGATTG	TTGCCTATGA	950
	TCGACTAATT	ACTGGATCTG	ATAAAATATGA	TTGGTATGTT	TCTTTTGATA	1000
	ATGAAAAAGT	TGGTGAATT	CAAGGTCTT	CACTTGCTGC	GGGTCTATTA	1050
	GGAAAAGAAG	ATGGTGCTT	TGATTCAATT	GATCAAATGA	ATGAATATCT	1100
5	AAAATCACAT	ATGCCCAAG	AGACAATTTC	TTTTTATACA	ATCGCGGGTT	1150
	CCCAAGATGA	TAATAATTCC	CAATATTTT	ATAATGGTGC	AATGAAAGTA	1200
	CTTAAAGAAT	TAATGAAAAA	TTCGCAAAT	AAAATAATTG	ATTATCTCC	1250
	TGAAGGCAGA	AATGCTGTT	ATGTCCCAGG	ATGAAATTAT	GGAACTGCCG	1300
	GTCAAAGAAT	CCAATCTTT	CTAACAAATTA	ACAAAGATCC	AGCAGGTGGT	1350
10	AATAAAATCA	AAGCTGTTGG	TCAAAACCA	GCTTCTATTT	TCAAAGGATT	1400
	TCTTGGCCCCA	AATGATGGAA	TGGCCGAACA	AGCAATCACC	AAATTAAAAC	1450
	TTGAAGGGTT	TGATACCCAA	AAAATCTTG	TAACTCGTCA	AGATTATAAT	1500
	GATAAAGCCA	AAACTTTAT	CAAAGACGGC	GATCAAATA	TGACAATTAA	1550
	TAAACCTGAT	AAAGTTTAG	GAAAAGTTGC	TGTTGAAGTT	CTTCGGGTTT	1600
15	TAATTGCAAA	GAAAAATAAA	GCATCTAGAT	CAGAAGTCGA	AAACGAACTA	1650
	AAAGCAAAAC	TACCAAATAT	TTCATTTAAA	TATGATAATC	AAACATATAA	1700
	AGTACAAGGT	AAAAATATTA	ATACAATTTC	AGTAAGTCCA	GTAATTGTTA	1750
	CAAAAGCTAA	TGTTGATAAT	CCTGATGCCT	AA		1782

20 Accordingly, in a further aspect the present invention provides an isolated nucleic acid fragment encoding a putative protective antigen against Mycoplasma hyopneumoniae or related infections, said nucleic acid fragment:

	10	20	30	40	50	
25	1234567890	1234567890	1234567890	1234567890	1234567890	
	ATGAAAAAAA	TGCCACTATA	CCAGAGGAAA	GAGCAGTATA	AAAAATAATT	50
	AAAATTACAT	TTTCTTCATT	TGCGCCAGAA	TTTTTAAGAA	TTAGTACATT	100
	AAAAAGTAGA	ACAAAAGTTA	TTAATGTAAA	CATTAGCGCA	ATCCTTAAGA	150
30	AAAAATTAAA	AGTTTTATCT	ATTTTTTTA	ATCGAAATCC	AACCAGGCAT	200
	AAATCTTGT	CAGTATTAT	CAAGTCGGTA	TTTTTTCATT	ATTCTACTA	250
	AAATATTATT	TGAATTGCA	TTTCCATAA	TCTAAAATT	TACATTTTT	300
	TATAACAATT	TTTAAAAATT	ACTCTTTAAT	TTATAGTATT	TTTTTATTTT	350
	TTAGTCTAAA	TTATAAAATT	ATCTTGAATT	TTATTTGAAT	TTTTATAATT	400
35	TAGTACTAAA	AAATACAAAT	ATTTTTTCT	ATTCTAAGAA	AAATTCAATT	450
	TTTAAAAAAA	ATTGATTTT	ATAGTATAAT	TTGTTTGTAT	AATTGAATTA	500
	ACTTGATTTG	AAAGGGAAACA	AAATGAAAAA	AATGCTTAGA	AAAAAATTCT	550
	TGTATTCATC	AGCTATTAT	GCAACTTCGC	TTGCATCAAT	TATTGCATT	600
	GTTGCAGCAG	GTTGGGACA	GACAGAATCA	GGTTCACCTT	CTGATTCTAA	650
40	ACCACAAGCC	GAGACGCTAA	AACATAAAAGT	AAGTAATGAT	TCTATTGAA	700
	TAGCACTAAC	CGATCCGGAT	AATCCTCGAT	GAATTAGTGC	CCAAAAAAGAT	750
	ATTATTCTT	ATGTTGATGA	AACAGAGGCA	GCAACTTCAA	CAATTACAAA	800
	AAACCAGGAT	GCACAAAATA	ACTGACTCAC	TCAGCAAGCT	AATTAAAGCC	850
	CAGGCCAAA	AGGATTATT	ATTGCCCTG	AAAATGGAAG	TGGAGTTGGA	900
45	ACTGCTGTTA	ATACAATTGC	TGATAAAAGGA	ATTCCGATTG	TTGCCTATGA	950
	TCGACTAATT	ACTGGATCTG	ATAAAATATGA	TTGGTATGTT	TCTTTTGATA	1000
	ATGAAAAAGT	TGGTGAATT	CAAGGTCTT	CACTTGCTGC	GGGTCTATTA	1050
	GGAAAAGAAG	ATGGTGCTT	TGATTCAATT	GATCAAATGA	ATGAATATCT	1100
	AAAATCACAT	ATGCCCAAG	AGACAATTTC	TTTTTATACA	ATCGCGGGTT	1150
50	CCCAAGATGA	TAATAATTCC	CAATATTTT	ATAATGGTGC	AATGAAAGTA	1200
	CTTAAAGAAT	TAATGAAAAA	TTCGCAAAT	AAAATAATTG	ATTATCTCC	1250
	TGAAGGCAGA	AATGCTGTT	ATGTCCCAGG	ATGAAATTAT	GGAACTGCCG	1300
	GTCAAAGAAT	CCAATCTTT	CTAACAAATTA	ACAAAGATCC	AGCAGGTGGT	1350

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	AATAAAATCA	AAGCTGTTGG	TCACAAACCA	GCTTCTATT	TCAAAGGATT	1400
	TCTTGCCCCA	AATGATGGAA	TGGCCGAACA	AGCAATCACC	AAATTAAAAC	1450
	TTGAAGGGTT	TGATACCCAA	AAAATCTTG	TAACTCGTCA	AGATTATAAT	1500
	GATAAAGCCA	AAACTTTTAT	CAAAGACGGC	GATCAAATA	TGACAATT	1550
5	TAAAACCTGAT	AAAGTTTAG	GAAAAGTTGC	TGTTGAAGTT	CTTCGGGTTT	1600
	TAATTGCAAA	AAAAAATAAA	GCATCTAGAT	CAGAAGTCGA	AAACGAAC	1650
	AAAGCAAAAC	TACCAAATAT	TTCATTAAA	TATGATAATC	AAACATATAA	1700
	AGTACAAGGT	AAAATATTA	ATACAATTT	AGTAAGTCCA	GTAATTGTTA	1750
	CAAAAGCTAA	TGTTGATAAT	CCTGATGCCT	AA		1782

10

As cross protection between various Mycoplasma such as M. hyorhinis and M. synoviae has been documented, similar antigens may also be detected in other Mycoplasma species (Figure 1).

In a still further aspect the present invention provides a method for preventing Mycoplasma infection in animals. Preferably the Mycoplasma disease is a Mycoplasma hyopneumoniae disease such as swine enzootic pneumonia (SEP). This method includes administering to an animal an effective amount of at least one protective antigen against Mycoplasma as described above.

The present invention further provides a vaccine composition including a prophylactically effective amount of at least one putative protective antigen against a Mycoplasma as herein described. Preferably the veterinary composition includes two or more putative protective antigens as herein described.

Accordingly in a preferred aspect the present invention provides a vaccine composition including two or more putative protective antigens selected from antigens having approximate molecular weights of 110-114, 90-94, 72-75, 60-64, 52-54 and 46-48 kilodaltons.

The vaccine composition may include any combination of two or more putative protective antigens selected from antigens having approximate molecular weights of 110-114, 90-94, 72-75, 60-64, 52-54 and 46-48 kD. The two or more antigens may be selected from antigens falling within one of the specified approximate molecular weights and/or antigens from different specified approximate molecular weights. The composition may contain 3, 4, 5 or 6 antigens selected from protective antigens having molecular weights of approximately 110-114, 90-94, 72-75, 60-64, 52-54 and 46-48 kD.

The vaccine compositions according to the present invention may be

administered orally or may be administered parenterally (for example by intramuscular, subcutaneous, intradermal or intravenous injection). The amount required will vary with the antigenicity of the active ingredient and need only be an amount sufficient to induce an immune response typical of existing vaccines.

5 Reactive experimentation will easily establish the required amount. Typical initial doses of vaccine or veterinary compositions may be approximately 0.001-1 mg active ingredient/kg body weight. The dose rate may increase or multiple doses may be used as needed to provide the desired level of protection.

The vaccine composition according to the present invention may further
10 include a veterinary acceptable carrier, diluent or excipient therefor. Preferably the active ingredient may be suspended or dissolved in a carrier. The carrier may be any solid or solvent that is nontoxic to the animal and compatible with the active ingredient. Suitable carriers include liquid carriers, such as normal saline and other nontoxic salts at or near physiological concentrations, and solid
15 carriers, such as talc or sucrose.

Preferably the vaccine contains an adjuvant, such as Freund's adjuvant, complete or incomplete, or immunomodulators such as cytokines may be added to enhance the antigenicity of the antigen if desired.

More preferably the adjuvant is of the mineral-oil type as these have been
20 found to be consistently superior at inducing antibody titres and Delayed Type Hypersensitivity responses. A particularly preferred adjuvant is that marketed under the trade designation Montanide ISA-50 and available from Seppic, Paris, France.

When used for administering via the bronchial tubes, the vaccine is
25 suitably present in the form of an aerosol.

In a still further aspect of the present invention there is provided a diagnostic kit including a diagnostic antigen against a Mycoplasma, preferably Mycoplasma hypneumoniae, identified and purified as described above.

The putative protective antigens according to the present invention may be
30 isolated and identified utilising the general methods described in Australian patent application 49035/90, the entire disclosure of which is incorporated herein by reference.

Accordingly, in a further aspect, the present invention provides a method for producing at least one antibody against a Mycoplasma. This method includes providing a biological sample taken a short time after an immune animal has been challenged with a Mycoplasma or Mycoplasma extract taken from the 5 infection site or an area of a lesion or an area close to the infection site or lesion; isolating cells from the biological sample; culturing cells in vitro in a suitable culture medium; and harvesting antibodies produced from said cells.

The Mycoplasma may be Mycoplasma hyopneumoniae.

10 The animal may be a mammal including humans. The mammal may be a domestic animal such as a pig, sheep or cattle.

The biological animal sample may be of any suitable type. The biological sample may be taken from animal tissue, organs, lymph or lymph nodes. The biological sample may be taken from the infection site, the lungs of the animal, or 15 an area of a lesion which may be formed or an area close to the infected site or a lesion such as in the lymph nodes draining from the lungs.

However, serum/plasma samples are not used as the biological samples according to this aspect of the present invention. It has been found that the majority of antibodies found in a serum/plasma sample are irrelevant to protection 20 or specific diagnosis or a Mycoplasma or are unrelated to the Mycoplasma. In addition, other serum/ plasma components may interfere with the specific reactions between pathogen components and antibodies to them.

In contrast, the probes described in the present invention are highly enriched in Mycoplasma-specific antibodies of particular importance to protective 25 immunity.

It is preferred that the biological samples are taken from the animals at a predetermined time in the development of the disease. In general, for a Mycoplasma infection, it has been found that the biological samples should be taken approximately 2 to 7 days after challenge with or after administration of 30 products obtained from a pathogen or with the pathogen itself.

The cells isolated from the biological sample may include B cells.

Thus, preferably the cells are taken a short time after in vivo stimulation.

preferably within approximately 2 to 5 days thereafter, resulting in the in vivo induction of antibody forming cells which will secrete specific antibodies into the culture medium after in vitro incubation.

In vitro secretion of antibodies in the culture medium by recently activated
5 B cells may be enhanced by the addition of helper factors to the cultures. The helper factors may be cytokines used alone or in combination, including Interleukin 1, 2, 3, 4, 5, 6, 7 and 8, colony stimulating factors, interferons and any other factors that may be shown to have an enhancing effect on specific B cell secretion.

10 The method of producing an antibody may include a further step of activating the cells isolated to proliferate and secrete and/or release antibodies.

The cell activation step may include adding a cell activating agent to the culture medium. The cell activating agent may be selected from mitogens and helper factors produced by leukocytes, or their synthetic equivalents or
15 combinations thereof.

The mitogens may be selected from the group including products derived from pokeweed (*Phytolacca americana*) also known as pokeweed mitogen (PWM), polyvinylpyrrolidone (PVP), polyadenylic-polyuridylic acid (poly(A-U)), purified protein derivate (PPD), polyinosinic-polycytidilic acid (poly(I-C)),
20 lipopolysaccharide (LPS), staphylococcal organisms or products thereof, Bacto-streptolysin O reagent (SLO), Staphylococcal phage lysate (SPL), Epstein-Barr virus (EBV), Nocardia water-soluble mitogen (NWEM), phytohemagglutinin (PHA), Concanavalin A (Con A), and dextran-sulphate and mixtures thereof. The cell proliferation agent may be any agent that indirectly or directly results in B cell
25 proliferation and/or antibody secretion such as solid-phase anti-immunoglobulin. The helper factors may be selected from the group including cytokines including interleukin 1, 2, 3, 4, 5, 6, 7 and 8, colony stimulating factors, interferons and any other helper factors that may be shown when added alone, or in combination with other factors and agents, to have an enhancing effect on specific B cell
30 proliferation and/or antibody secretion. This in no way is meant to be an exhaustive list of mitogens and cell actuating agents including helper factors.

The in vitro culturing of the cells may be conducted with or without prior

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steps to separate sub-populations of cells. The harvesting of antibodies may be conducted by harvesting of the supernatant from the culture medium. This supernatant contains antibodies secreted by these cells during the in vitro culture or artificially released from the B cells, for example by lysis of the B cells. It has
5 been found that the antibody-containing supernatants may be used directly to detect antigens of the Mycoplasma.

In a preferred aspect of the present invention, there is provided a method for identifying an antigen associated with a Mycoplasma, preferably Mycoplasma hyopneumoniae. This method includes

- 10 providing
a sample of a Mycoplasma; and
an antibody probe including at least one antibody against a
Mycoplasma;
probing the Mycoplasma sample with the antibody probe to detect at least
15 one antigen; and
isolating the antigen detected.

The sample of Mycoplasma may be mixed with a standard buffer solution and placed on a standard support such as an SDS-polyacrylamide gel to separate the proteins contained thereon (Figure 2).

- 20 Alternatively, the proteins may be selected utilising the non-ionic detergent Triton X-114 (TX-114). Insoluble material may be removed by centrifugation. Proteins soluble in the TX-114 phase may then be precipitated out (Figure 2).

The separate proteins may then be transferred to nitrocellulose, nylon or other sheets.

- 25 The probing with a suitable antibody may further include subjecting the product produced thereby to a detection assay. The detection assay may include Western blot techniques. The detection assay may be an immunoprecipitation assay, a radioimmunoassay, an enzyme-linked immunoassay or immunofluorescent assay (Figures 3, 4 and 5).

- 30 The antibody produced as described above may be utilized simply in the form of the supernatant harvested from the culture medium. Alternatively, the antibodies may be separated and purified.

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In a further preferred aspect of the present invention the antibody contained in the culture medium may be used for the affinity purification, preferably immuno-affinity purification of antigen.

Accordingly, in a preferred aspect there is provided a method for purifying
5 antigen. This method includes

providing

a crude antigen mixture; and

an antibody against a Mycoplasma immobilized on a suitable support;

10 subjecting the crude antigen mixture to affinity chromatography utilizing the immobilized antibody; and

isolating the purified antigen so formed.

The antibody is produced by the method described above.

Antibody can be obtained from the culture supernatant probe by
15 conventional methods. For example, methods usually used to purify immunoglobulins from serum or plasma, e.g. precipitation with ammonium sulphate, fractionation with caprylic acid, ion exchange chromatography, or by binding and elution from immobilized protein G or protein A, may be utilized. Antibody so obtained can then be coupled to suitable supports, e.g., CNBr-
20 activated Sepharose 4B (Pharmacia), Affi-gel (Bio-RAD), or other affinity chromatography supports able to bind proteins.

Immobilized antibody can then be applied to the fractionation and purification of specific antigen from a complex Mycoplasma extract by affinity chromatography. After binding of antigen to immobilized antibody, unbound
25 macromolecular species can be washed away from the solid support with, e.g. buffers containing 1.5 M NaCl. Subsequently the antigen can be eluted from the affinity column with, e.g. low or high pH buffer or buffers containing chaotropic ions, e.g. 0.5-3.0 M sodium thiocyanate.

The application of the antibody probe to affinity chromatography enables
30 sufficient quantities of specific antigens to be rapidly isolated from a complex crude extraction mixture for biochemical characterization, amino-acid sequencing and vaccination of animal for limited protection studies. Application of affinity

chromatography for obtaining antigen(s) avoids the difficulties often encountered when applying conventional biochemical techniques to the purification of an antigen about which little or no data is known. It also obviates the need to raise polyclonal or monoclonal antibodies for the purpose of "analytical" affinity chromatography. Large scale preparation may, however, require the preparation of polyclonal or monoclonal antibodies.

Having identified the antigen(s) molecular biology, chemical techniques, e.g. cloning techniques, may be used to produce unlimited amounts of this antigen or, alternatively, synthetic peptides corresponding to different fragments of the identified antigens may be used as a means to produce a vaccine.

Accordingly in a preferred aspect of the present invention there is provided a method for preparing a synthetic antigenic polypeptide against Mycoplasma, preferably Mycoplasma hypneumoniae, which method includes providing

a cDNA library or genomic library derived from a sample of Mycoplasma; and

an antibody probe as described above;

generating synthetic polypeptides from the cDNA library or genomic library;

probing the synthetic polypeptides with the antibody probe; and

isolating the synthetic antigenic polypeptide detected thereby.

Either cDNA or genomic libraries may be used. The cDNA or genomic libraries may be assembled into suitable expression vectors that will enable transcription and the subsequent expression of the clone cDNA, either in prokaryotic hosts (e.g. bacteria) or eukaryotic hosts (e.g. mammalian cells). The probes may preferably be selected from

- (i) synthetic oligonucleotide probes based on the amino acid sequence of the antigen identified and purified as described above;
- (ii) antibodies obtained from the culture medium produced as described above;
- (iii) monoclonal or polyclonal antibodies produced against the antigens identified and purified as described above;
- (iv) recombinant or synthetic monoclonal antibodies or polypeptides with

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specificity for the antigen, e.g. as described by Ward et al., Nature, 241, pages 544-546 (1989).

The synthetic antigenic polypeptide produced in accordance with the invention may be a fusion protein containing the synthetic antigenic peptide and
5 another protein.

In a further aspect of the present invention there is provided a DNA fragment encoding a putative protective antigen against Mycoplasma or related infections, said DNA fragments having a nucleic acid sequence according to
10 Figure 6a and 6b or an homologous sequence and functionally active fragments thereof.

In a further preferred aspect of the present invention there is provided a clone including a DNA fragment encoding a putative protective antigen against
15 Mycoplasma or related infections, said DNA fragments having a nucleic acid sequence according to Figure 6a and 6b or an homologous sequence and functionally active fragments thereof.

Preferably the clone is pC1-2.

20

The present invention will now be more fully described with reference to the accompanying Examples and drawings. It should be understood, however, that the description following is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

25

IN THE FIGURES:

FIGURE 1: SDS-Polyacrylamide gel (12.5%) profiles of SDS extracts of species of mycoplasma- Coomassie R250 stained.

30

- Lane 1 Pre-stained Molecular Weight Standards.
Lane 2 *M. gallisepticum*.

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- Lane 3 *M. synoviae*.
Lane 4 *M. hyopneumoniae*.
Lane 5 *M. hyorhinis*.
Lane 6 *M. flocculare*.

5

FIGURE 2: SDS-Polyacrylamide gel (12.5%) profiles of extracts of strains of *M. hyopneumoniae* - Coomassie R250 stained gel

- Lane 1 Pre-stained Molecular Weight Standards.
10 Lane 2 Triton X-114 extract of *M. hyopneumoniae* - strain Beaufort.
Lane 3 As for Lane 2.
Lane 4 SDS extract of *M. hyopneumoniae* strain Beaufort.
Lane 5 SDS extract of *M. hyopneumoniae* strain 10110.

- 15 FIGURE 3: Western blots of Triton X-114 extracted antigens from *M. hyopneumoniae* strain Beaufort, probed with serum and supernatant antibody probes.

- Lane 1 No antibody control.
20 Lane 2 Dookie pig serum control 1/200.
Lane 3 Pig 105 supernatant.
Lane 4 Pig 1 supernatant.
Lane 5 Dookie pig supernatant.

- 25 FIGURE 4: Western blots of SDS extracted antigens from *M. hyopneumoniae* strain Beaufort probed with paired serum and supernatant antibody probes.
Fractionation of antigens on SDS Polyacrylamide gel (12.5%).

- Lane 1 a) Pig 453 supernatant.
30 b) Pig 453 serum 1/100.
Lane 2 a) Pig 105 supernatant.
 b) Pig 105 serum 1/100.

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- Lane 3 a) Pig 1 superanatant.
 b) Pig 1 serum 1/100.
Lane 4 a) Pig 15 supernatant.
 b) Pig 15 serum 1/100.
5 Lane 5 a) Dookie supernatant.
 b) Dookie serum 1/100.
Lane 6 No antibody control.

FIGURE 5: Western blots of SDS extracted antigens from *M. hyopneumoniae* strain Beaufort probed with paired serum and supernatant antibody probes. Fractionation of antigens on SDS Polyacrylamide gel (10.0 %).

- Lane 1 a) Pig 453 supernatant.
 b) Pig 453 serum 1/100.
15 Lane 2 a) Pig 105 supernatant.
 b) Pig 105 serum 1/100.
Lane 3 a) Pig 1 supernatant.
 b) Pig 1 serum 1/100.
Lane 4 a) Pig 15 supernatant.
 b) Pig 15 serum 1/100.
20 Lane 5 a) Dookie supernatant.
 b) Dookie serum 1/100.
Lane 6 No antibody control.

25 **FIGURE 6:** The entire 48 k gene sequence.

FIGURE 7: THE 48kDa protein sequence of the 48k gene sequence.

- 15 -

EXAMPLE 1

Mycoplasma hyopneumoniae media

Friss Media

- 5 Hovind-Hougen, K., Friss, N.F., Research in Veterinary Science, 1991, 51,
pp 155-163, "Morphological & Ultrastructural Studies of M flocculare and M
hyopneumoniae in vitro".

250 ml Hanks BSS

10 140 ml Water

1.5 gm Brain Heart infusion

1.6 gm PPLO Broth w/o CV

Autoclave at 120°C for 20 minutes

18 ml Yeast Extract (100g YSC-2 Sigma in 750 ml)

15 3.7 ml 0.2% DNA in 0.1% Na₂CL₃

5.14 ml 1% -NAD

0.6 ml 1% Phenol red

Adjust to pH 7.3 to 7.4

20 Filter through 0.45 um, 0.2 um membrane, store at 4°C.

Add sterile Horse or Pig serum to 20%

and Antibiotics prior to use

Etheridge Media

- 25 Etheridge, J.R., Cottew, G.S., Lloyd, L.C., Australian Veterinary Journal,
1979, August 55, pp 356-359, "Isolation of Mycoplasma hyopneumoniae from
lesions in experimentally infected pigs".

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	<u>Materials</u>	<u>For 600 mls</u>
	Hanks BSS	18.9 ml
	Hartleys Digest broth	1.28 gm
5	Heart Infusion broth	1.65 gm
	Lactalbumin hydrolysate	2.21 gm
	Glucose	4.41 gm
	Yeast Extract autolysate	8.82 ml
	Pig Serum (filtered)	163 ml
10	1% NAD	6.17 ml
	1% Phenol red	1.32 ml
	0.2% DNA in 0.1% Na ₂ CO ₃	4.41 ml

Make up to 600 ml with MQ water (about 350 - 400 ml)

- 15 Adjust pH to 7.4 and filter through: 3.0 um, 0.8 um, 0.45 um, 0.2 um.
 Store at 4°C.

Development of Immune Sows

- Cull sows and naive gilt (unmated sow designated Dookie).
 20 Challenged on numerous occasions, with culture grown M. hyopneumoniae and lung homogenate. Given intranasally and intratracheally. Period of challenge - from September, 1991 to 21st January, 1992.

Tiamulin antibiotic given 31st January, 1992 to 4th February, 1992.
 Rested for approximately 8 weeks.

25 Infectious Challenge

- 120 ml of frozen culture of M. hyopneumoniae strain Beaufort, spun down (12,000 xg, 20 min.) and resuspended in 50 ml complete medium and cultured overnight at 37°C. The overnight culture was centrifuged (12,000 xg, 20 min.) and the Mycoplasma cells resuspended in 10 ml serum free Mycoplasma culture medium. The 10 ml of concentrated mycoplasma was administered to anaesthetised immune sows via a catheter to ensure the inoculum was placed into the trachea.

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Three of four days post-challenge, the sows were killed, and lymph nodes draining the lungs taken - these included the left and right tracheobronchial lymph nodes, and the lymph nodes located at the bifurcation of the trachea.

Antibody probes were prepared from pig lymph nodes and utilised to detect 5 putative protection antigens as described in Australian Patent Application 49035/90 referred to above. Separate cell cultures were obtained from individual lymph nodes. Culture supernatants were harvested after 5 days of culture.

Antigen Preparation

Mycoplasma hyopneumoniae strain Beaufort was cultured in Etheridge 10 media until the pH had dropped to between 6.8 and 7.0. Cells of M. hyopneumoniae were harvested from culture by centrifugation at 12,000 xg for 20 min., washed 4 times with either sterile PBS or 0.25 M NaCl and then the pelleted cells extracted with one of the following.

(i) Sodium dodecyl sulphate (SDS)

15 The cell pellet was resuspended in 0.2% SDS and extracted for 2 hours at 37°C. Insoluble material was pelleted from the extract at 12,000 xg for 10 min. and the soluble extract run on SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

(ii) Triton X-114

20 The method of Bordier (J. Bio. Chem. 1981, 256:1604-1606) was used to selectively extract membrane proteins using the non-ionic detergent Triton X-114.

The cell pellet was resuspended in cold PBS to 2 mg/ml protein and a cold pre-condensed solution of TX-114 added to give a final concentration of 1% (v/v) TX-114. Extraction was achieved by incubation overnight at 4°C with gentle 25 mixing. Insoluble material was removed by centrifugation at 12,000 xg for 20 min. at 4°C. The Triton X-114 soluble membrane proteins were then obtained by achieving a phase separation at 37°C.

Proteins soluble in TX-114 phase were precipitated with 80% ethanol in the presence of carrier dextran (80,000 molecular weight) at -70°C overnight. The 30 proteins were collected by centrifugation at 12,000 xg for 30 min. and dissolved to 500 ug/ml in 4 M urea.

Identification of Antigens

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Six antigens were identified utilising the above- mentioned technique. The identified antigens were those that were consistently identified by the antibody probes from the immune cultures and the Dookie gilt. The results are summarised in Table 1.

5

TABLE 1

	<u>Molecular Weight (kD)</u>	<u>Characteristics</u>
	110-114	SDS Extracted
	90-94	SDS Extracted
10	72-75	Triton X-114 Extracted
	60-64**	SDS Extracted. Partitions to aqueous phase of Triton X-114 extract.
	52-54	Triton X-114 Extracted
	46-48	Triton X-114 Extracted

15

** Two antigens of approximate molecular weight 62 kD were identified.

<u>Molecular Weight (kD)</u>	<u>Amino Acid Sequence</u>
------------------------------	----------------------------

20	46-48	48 K N-Terminal: AGXGQTESGSTSDSKPQAETLKHKV 48 K CNBR F 1: TIYKPDVKLGKVAVEVLRVLIAKKNKASR 48 K CNBR F 2: AEQAITKLKLEGFDTQ 48 K CNBR F 3: KNSQNKIIDLSPPEG
25	52-54	52 K N-Terminal: AGXWAKETTKEEKS 52 K CNBR F 1: AWVTADGTVN 52 K CNBR F 2: AIVTADGTVNDNKPNQWWRKY
30	60-64	62 K N-Terminal: MKLAKLLKGFX (N/L)(M/V) IK
	60-64	62 K N-Terminal ADP(F/I)(R/E)Y(V/A)PQG(Q/A)X(M/N)VG

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72-75 74 K N-Terminal: AGXLQKNSLLEEVWYLA
 74 K CNBR F 1: AKNFDFAPSIQGYKKIAHEL
 74 K CNBR F 2: NLKPEQILQLLG
5 74 K CNBR F 3: LLKAEXNKXIEEINTXLDN

CNBR - Cyanogen Bromide fragment

X denotes an undetermined amino acid

(A/B) - residue may be A or B

10

PCR of 48kDa Gene

Polymerase Chain Reaction (PCR) oligonucleotide primers were designed from the amino acid sequences obtained from the N-terminal and internal cyanogen bromide (CNBr) derived peptides. Inosine (I) was substituted at 15 positions of high redundancy. The following primers were used in a standard PCR assay, run on a Bartelt Gene Machine Robotic thermal cycling instrument.

Oligo 48 K CNBr F 1: ACIAACGACGAGAAGCCICAGGC

T T A A A

20 Oligo 48 K CNBr F 2: TTIAGCTTGTGATIGCCTGCTC

AT A T T
T

Oligo 48 K CNBr F 3: AGGTCGATGATCTTCCAICC

25 AA A A T T
T T

The resulting PCR products were visualised on a 1.5% agarose gel, excised, and purified using Prep-a-Gene (BioRad). They were cloned by standard techniques 30 into a dideoxy tailed T-vector (Holton and Graham, Nucleic Acids Research 19: 1156, 1991) and the nucleic acid sequence determined. The PCR product, obtained from the reaction using primers F1 and F2 shown above, was of

- 20 -

approximately 810 base pairs and was shown by sequencing to code for the previously determined amino acid sequence of the purified native 46-48kDa protein.

5 Genomic clone isolation at 48 k gene

The entire 48k gene and 48kDa protein (Figures 6 and 7) has been isolated and sequenced. The gene was obtained from an *M. hyopneumoniae* genomic library made by digesting genomic DNA with the restriction enzyme CLA I and ligating the fragments into the vector pBluescript (Stratagene). The ligated product was then electroporated into *Escherichia coli* strain SURE (Stratagene) and the cells plated on Luria Broth agar plates containing 100 µg/ml Ampicillin (LB-Amp). The library was screened by DNA hybridisation with a polymerase chain reaction (PCR) product specific for the 48 kDa protein. Positive clones were grown in LB-Amp, the cells harvested and the DNA isolated and partially sequenced for confirmation.

The positive clone pC1-2 was entirely sequenced and the protein sequence deduced. This was compared to the protein sequence obtained from the N terminus and Cyanogen Bromide fragments of the 48 kDa protein to show the that the gene encoded the desired protein.

Adjuvant Selection

Young piglets, 5-7 weeks of age, were immunised with identified antigen(s). The antigens include Triton X-114 extract and identified proteins of 46-48, 52-53, 60-64, 70-75, 90-94 and 110-114 kD, either singly or in combination. An immunising dose of antigen, containing between 5-100 µg protein, was given by intramuscular injection in combination with an adjuvant. An adjuvant is selected from

- (i) Seppic Montanide ISA-50
- 30 (ii) Quill A and other derivatives of saponin,
- (iii) oil in water emulsion employing a mineral oil such as Bayol F/Arlacel A,
- (iv) oil in water emulsion employing a vegetable oil such as corn oil.

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- saflower oil or other with lecithin as emulsifier,
- (v) aluminium hydroxide gel, and
- (vi) nonionic block polymer such as Pluronic F-127 produced by BASF (U.S.A.).

5 Immunising doses were given at 2-4 week intervals, the number of doses being dependent on the adjuvant and amount of antigen, but preferably 2 to 3 doses are given.

10 Adjuvants were treated on the basis of being able to induce antibody titres, as measured by ELISA, and by assessment of induced cell-mediated immunity as tested by Delayed-Type Hypersensitivity (DTH) reaction.

The results clearly show that mineral-oil type adjuvants are consistently superior at inducing antibody titres and DTH responses (Table 2). In particular an adjuvant marketed under trade designation Montanide ISA-50 and available from Seppic, Paris, France has been found to be suitable.

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TABLE 2

GROUP	Animal Number	DTH 24 Hour Response	DTH 48 Hour Response	Antibody (450 nm) Levels
CONTROL (Unvaccinated)	19	0	0	0.061
	11	0	0	0.010
	1	-	-	0.005
	15	0	0	0.038
	7	0	0	0.005
QUIL A	18	+	0	0.753
	25	+	0	0.788
	17	0	0	0.638
	168	-	±	0.642
VEG. OIL	169	+++	0	0.316
	22	0	0	0.621
	4	+	0	0.666
	6	+	+	0.239
	13	+++	++	0.457
MIN. OIL	14	+++	++	1.086
	5	+++	++	1.024
	23	+++	+	0.864
	16	+++	0	0.975
	21	+	±	0.954

5 TABLE 2: Antibody levels and DTH responses in pigs measured 2 weeks after the third injection of antigen from M. hyopneumoniae. (- = no response; ± = faint reddening; + = faint reddening and swelling; ++ = reddening; +++ = swelling with or without reddening).

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Protection Pen Trial

Groups of 9 young piglets, 6 weeks of age, were immunised with purified and semi-purified antigens as shown in Table 3 below. The antigens were purified on reversed-phase HPLC using a formic acid solvent system with an
5 acetonitrile gradient.

Antigens were resolubilised in 4 Molar urea before incorporation in mineral oil adjuvant.

The immunisation schedule is as shown in Table 2.

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TABLE 3

Protocol for Pen Trial of Antigens of *Mycoplasma Hyopneumoniae*

5 VACCINATIONS & BLEEDS

<u>Treatment</u>	<u>Day Number</u>
1st Vaccination	0
2nd Vaccination	14
3rd Vaccination	50
Infectious Challenge	64
Slaughter	91

ANTIGEN DOSES

Partly Purified 62 kD	1st & 2nd Vaccns. 50µg COMPLEX ANTIGEN/DOSE 3rd Vaccn. - 220µg PARTIALLY PURIFIED ANTIGEN/DOSE
(Purified)74+52kD	1st Vaccn. 20µg total protein/DOSE 2nd Vaccn. 13µg total protein/DOSE 3rd Vaccn. 17µg total protein/DOSE
(Purified) 48KD	1st Vaccn. 20µg/DOSE 2nd Vaccn. 18µg/DOSE 3rd Vaccn. 27µg/DOSE

**10 ALL PROTEIN ESTIMATIONS DONE BY "BCA" PROTEIN ASSAY (Pierce,
Illinois, U.S.A.)**

Protection from infection with *Mycoplasma hyopneumoniae* was assessed by infectious challenge 2 weeks after the final immunisation. Infectious challenge 15 was achieved by intranasal administration of 10ml of a 10% (w/v) lung homogenate, prepared from infected lung, and by housing test piglets with

- 25 -

previously infected piglets. Four weeks after infectious challenge, the animals were killed and the extent and degree of lung lesions assessed (Table 4).

TABLE 4

5

Pen Trial of Antigens of Mycoplasma Hyopneumoniae

Group No.	No. Pneumonia	Median Lung	% Reduction
	Free (%)	Lesion Score	(from Median)
Controls	1 (11)	13	0%
62 kD	0 (0)	5	61%
74+52 kD	3 (33)	6.75	48%
48 kD	2 (22)	6.25	52%

REFERENCE

- Warren H.S. and Chedid, L.A., Future Prospects for Vaccine Adjuvants CRC
10 Critical Reviews in Immunology 8 : 83-108, 1988.

Finally, it is to be understood that various other modifications and/or alterations may be made without departing from the spirit of the present invention
15 as outlined herein.

CLAIMS:

1. A putative protective antigen against a Mycoplasma, prepared by a method including
 - 5 providing a sample of a Mycoplasma;
 - an antibody probe including at least one antibody against a Mycoplasma produced by a method including:
 - 10 providing a biological sample taken a short time after an immune animal has been challenged with a Mycoplasma or Mycoplasma extract taken from the infection site or an area of a lesion or an area close to the infection site or lesion;
 - isolating cells from the biological sample;
 - 15 culturing cells in vitro in a suitable culture medium; and harvesting antibodies produced from said cells;
 - probing the Mycoplasma sample with the antibody probe to detect at least one antigen; and isolating the antigen detected.
- 20 2. A putative protective antigen according to claim 1 wherein the Mycoplasma is Mycoplasma hyopneumoniae.
3. A putative protective antigen against Mycoplasma hyopneumoniae, or related infections, selected from the group of antigens having approximate molecular weights of 110-114, 90-94, 72-75, 60-64, 52-54 and 46-48 kilodaltons (kD), as herein described, mutants, derivatives and fragments thereof.
4. A putative protective antigen according to claim 3 which is a surface protein.

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5. A putative protective antigen according to claim 3 or 4 which is a surface lipo-protein or membrane protein.
6. A putative protective antigen according to any one of claims 3-5 having
5 approximate molecular weight of 110-114, 90-94, 74, 62, 52 and 48 kD.
7. A putative protective antigen according to claim 3 wherein the antigen in
the 72-75 kD region contains the following N-terminal amino acid sequence:

AGXLQKNSLLEEVWYAL

10

8. A putative protective antigen according to claim 7 further including one or
more of the following N-terminal amino acid sequences:

AKNDFAPSIQGYKKIAHEL

NLKPEQILQLLG

15

LLKAEXNKXIEEINTXLDN

9. A putative protective antigen according to claim 3 wherein the antigen in
the 60-64 kD region contains the following N-terminal amino acid sequence:

MKLAKEKGFX(N/L)(M/V)IK

20

ADP(F/I)(R/E)Y(V/A)PQG(Q/A)X(M/N)VG

10. A putative protective antigen according to claim 3 wherein the antigen in
the 52-54 kD region contains the following N-terminal amino acid sequence:

AGXWAKETTKEEKS

25

11. A putative protective antigen according to claim 10 further including one or
more of the following N-terminal amino sequences:

AWVTADGTVN

AIVTADGTVNDNKPNQWVRKY.

30

12. A putative protective antigen according to claim 3 wherein the antigen in
the 46-48 kD region contains the following N-terminal amino acid sequence:

AGXGQTESGSTSDSKPQAETLKHKV

13. A putative protective antigen according to claim 12 further including one or more of the following internal amino acid sequences:

5 TIYKPDVKVLGVAVEVLRVLIAKKNKASR
 AEQAITKLKLEGFDTQ
 KNSQNKIIDLSP EG

14. An isolated nucleic acid fragment encoding a putative protective antigen
 10 against Mycoplasma hyopneumoniae or related infections, said nucleic acid
 fragment including the following sequence, mutants, derivatives, recombinants
 and fragments thereof:

	10	20	30	40	50	
15	1234567890	1234567890	1234567890	1234567890	1234567890	
	ATGAAAAAAA	TGCCACTATA	CCAGAGGAAA	GAGCAGTATA	TAAAATAATT	50
	AAAATTACAT	TTTCTTCATT	TGCGCCAGAA	TTTTTAAGAA	TTAGTACATT	100
	AAAAGTAGA	ACAAAAGTTA	TTAATGTAAA	CATTAGCGCA	ATCCTTAAGA	150
20	AAAATTAAA	AGTTTATCT	ATTTTTTTA	ATCGAAATCC	AACCAGGCAT	200
	AAATCTTGT	CAGTATTTAT	CAAGTCGGTA	TTTTTCATT	ATTTCTACTA	250
	AAATATTATT	TGAATTTGCA	TTTTCATCAA	TCTAAAATTT	TACATTTTTT	300
	TATAACAATT	TTTAAAATT	ACTCTTTAAT	TTATAGTATT	TTTTTATTTT	350
	TTAGTCTAAA	TTATAAAATT	ATCTTGAATT	TTATTTGAAT	TTTTATAATT	400
25	TAGTACTAAA	AAATACAAAT	ATTTTTTCCT	ATTCTAAGAA	AAATTCTATT	450
	TTTAAAAAAA	ATTGATTTTT	ATAGTATAAT	TTGTTTGTAT	AATTGAATTA	500
	ACTTGATTG	AAAGGGAACAA	AAATGAAAAA	AATGCTTAGA	AAAAAATTCT	550
	TGTATTCATC	AGCTATTTAT	GCAACTTCGC	TTGCATCAAT	TATTGCATTT	600
	GTTGCAGCAG	GTTGGGACAA	GACAGAATCA	GGTTCAACTT	CTGATTCTAA	650
30	ACCACAAGCC	GAGACGCTAA	AACATAAAGT	AAGTAATGAT	TCTATTGAA	700
	TAGCACTAAC	CGATCCGGAT	AATCCTCGAT	GAATTAGTGC	CCAAAAAGAT	750
	ATTATTTCTT	ATGTTGATGA	AACAGAGGCA	GCAACTTCAA	CAATTACAAA	800
	AAACCAAGGAT	GCACAAAATA	ACTGACTCAC	TCAGCAAGCT	AATTAAAGCC	850
	CAGCGCCAAA	AGGATTATT	ATTGCCCTG	AAAATGGAAG	TGGAGTTGGA	900
35	ACTGCTGTTA	ATACAATTGC	TGATAAAGGA	ATTCCGATTG	TTGCCTATGA	950
	TCGACTAATT	ACTGGATCTG	ATAAATATGA	TTGGTATGTT	TCTTTTGATA	1000
	ATGAAAAAGT	TGGTGAATT	CAAGGTCTTT	CACTTGCTGC	GGGTCTATTA	1050
	GGAAAAGAAG	ATGGTGCTTT	TGATTCAATT	GATCAAATGA	ATGAATATCT	1100
	AAAATCACAT	ATGCCCAAG	AGACAATTTC	TTTTTATACA	ATCGCGGGTT	1150
40	CCCAAGATGA	TAATAATTCC	CAATTTTT	ATAATGGTGC	AATGAAAGTA	1200
	CTTAAAGAAT	TAATGAAAAA	TTCGAAAAT	AAAATAATTG	ATTTATCTCC	1250
	TGAAGGCGAA	AATGCTGTTT	ATGTCCCAGG	ATGAAATTAT	GGAAGTGGCG	1300
	GTCAAAGAAT	CCAATTTT	CTAACAAATT	ACAAAGATCC	AGCAGGTGGT	1350
	AATAAAATCA	AAGCTGTTGG	TTCAAAACCA	GCTTCTATT	TCAAAGGATT	1400
45	TCTTGCCCCA	AATGATGGAA	TGGCCGAACA	AGCAATCACC	AAATTAAAAC	1450
	TTGAAGGGTT	TGATACCCAA	AAAATCTTG	TAACTCGTCA	AGATTATAAT	1500
	GATAAGCCA	AAACTTTAT	CAAAGACGGC	GATCAAATA	TGACAATT	1550

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	TAAACCTGAT	AAAGTTTAG	GAAAAGTTGC	TGTTGAAGTT	CTTCGGGTTT	1600
	TAATTGCAAA	GAAAATAAA	GCATCTAGAT	CAGAAGTCGA	AAACGAACTA	1650
	AAAGCAAAAC	TACCAAATAT	TTCATTTAAA	TATGATAATC	AAACATATAA	1700
	AGTACAAGGT	AAAAATATTA	ATACAATTTC	AGTAAGTCCA	GTAATTGTTA	1750
5	CAAAAGCTAA	TGTTGATAAT	CCTGATGCCT	AA		1782

15. An isolated nucleic acid fragment according to claim 14 encoding a putative protective antigen wherein the antigen is in the 46-48 kD region including the following nucleic acid sequence, mutants, derivatives, recombinants and
10 fragments thereof:

	10	20	30	40	50	
	1234567890	1234567890	1234567890	1234567890	1234567890	
15	ATGAAAAAAA	TGCCACTATA	CCAGAGGAAA	GAGCAGTATA	TAAAATAATT	50
	AAAATTACAT	TTTCTTCATT	TGCGCCAGAA	TTTTTAAGAA	TTAGTACATT	100
	AAAAAGTAGA	ACAAAAGTTA	TTAATGTAAA	CATTAGCGCA	ATCCTTAAGA	150
	AAAAATTAAA	AGTTTTATCT	ATTTTTTTA	ATCGAAATCC	AACCAGGCAT	200
	AAATCTTGT	CAGTATTAT	CAAGTCGGTA	TTTTTCATT	ATTTCTACTA	250
20	AAATATTATT	TGAATTGCA	TTTCCATAA	TCTAAAATT	TACATTTTTT	300
	TATAACAATT	TTTAAAAATT	ACTCTTTAAT	TTATAGTATT	TTTTTATTTT	350
	TTAGTCTAAA	TTATAAAATT	ATCTTGAATT	TTATTTGAAT	TTTTATAATT	400
	TAGTACTAAA	AAATACAAAT	ATTTTTCT	ATTCTAAGAA	AAATTCAATT	450
	TTTAAAAAAA	ATTGATTTT	ATAGTATAAT	TTGTTTGAT	AATTGAATTA	500
25	ACTTGATTTG	AAAGGGAAACA	AAATGAAAAA	AATGCTTAGA	AAAAAATTCT	550
	TGTATTTCATC	AGCTATTAT	GCAACTTCGC	TTGCATCAAT	TATTGCATT	600
	GTTGCAGCAG	GTTGTGGACA	GACAGAACATCA	GGTTCAACTT	CTGATTCTAA	650
	ACCACAAGCC	GAGACGCTAA	AACATAAAAGT	AAGTAATGAT	TCTATTGAA	700
	TAGCACTAAC	CGATCCGGAT	AATCCTCGAT	GAATTAGTGC	CACAAAAGAT	750
30	ATTATTTCTT	ATGTTGATGA	AACAGAGGCA	GCAACTTCAA	CAATTACAAA	800
	AAACCAGGAT	GCACAAAAATA	ACTGACTCAC	TCAGCAAGCT	AATTAAAGCC	850
	CAGCGCCAAA	AGGATTTATT	ATTGCCCTG	AAAATGGAAG	TGGAGTTGGA	900
	ACTGCTGTTA	ATACAATTGC	TGATAAAGGA	ATCCGATTG	TTGCCTATGA	950
	TCGACTAATT	ACTGGATCTG	ATAAATATGA	TTGGTATGTT	TCTTTGATA	1000
35	ATGAAAAAGT	TGGTGAATTA	CAAGGTCTTT	CACTTGCTGC	GGGTCTATTA	1050
	GGAAAAAGAAG	ATGGTGCCTT	TGATTCAATT	GATCAAATGA	ATGAATATCT	1100
	AAAATCACAT	ATGCCCAAG	AGACAATTTC	TTTTTATACA	ATCGCGGGTT	1150
	CCCAAGATGA	TAATAATTCC	CAATTTTTT	ATAATGGTGC	AATGAAAGTA	1200
	CTTAAAGAAT	TAATGAAAAA	TTCGCAAAAT	AAAATAATTG	ATTATCTCC	1250
40	TGAAGGCGAA	AATGCTGTTT	ATGTCCCAGG	ATGAAATTAT	GGAAC TGCCG	1300
	GTCAAAGAAT	CCAATCTTT	CTAACAAATTA	ACAAAGATCC	AGCAGGTGGT	1350
	AATAAAATCA	AAGCTGTTGG	TTCAAAACCA	GCTTCTATT	TCAAAGGATT	1400
	TCTTGCCCCA	AATGATGGAA	TGGCCGAACA	AGCAATCACC	AAATTAAAAC	1450
	TTGAAGGGTT	TGATACCCAA	AAAATCTTTG	TAACTCGTCA	AGATTATAAT	1500
45	GATAAAAGCCA	AAACTTTTAT	CAAAGACGGC	GATCAAATA	TGACAATTAA	1550
	TAACACCTGAT	AAAGTTTAG	GAAAAGTTGC	TGTTGAAGTT	CTTCGGGTTT	1600
	TAATTGCAAA	GAAAATAAA	GCATCTAGAT	CAGAAGTCGA	AAACGAACTA	1650
	AAAGCAAAAC	TACCAAATAT	TTCATTTAAA	TATGATAATC	AAACATATAA	1700
	AGTACAAGGT	AAAAATATTA	ATACAATTTC	AGTAAGTCCA	GTAATTGTTA	1750
50	CAAAAGCTAA	TGTTGATAAT	CCTGATGCCT	AA		1782

- 30 -

16. A method for producing an antibody against a Mycoplasma including providing a biological sample taken a short time after an immune animal has been challenged with a Mycoplasma or Mycoplasma extract taken from the infection site or an area of a lesion or an area close to the infection site or lesion;
- 5 isolating cells from the biological sample; culturing cells in vitro in a suitable culture medium; and harvesting antibodies produced from said cells.
17. A method according to claim 16 wherein the biological sample is taken at a predetermined time after the animal has been challenged with a Mycoplasma, preferably 2 to 7 days after challenge.
18. A method according to claim 16 wherein the culturing of cells in vitro further includes addition of helper factors to the culture, said helper factors selected from the group including cytokines used alone or in combination, including Interleukin 1, 2, 3, 4, 5, 6, 7 and 8, colony stimulating factors, interferons and any other factors that may be shown to have an enhancing effect on specific B cell secretion.
- 20 19. A method according to any one of claims 16-18 further including a cell activation step including activating the cells isolated to proliferate and secrete and/or release antibodies said cell activation step including adding a cell activating agent to the culture medium, said cell activating agent selected from the group including mitogens as herein described and helper factors produced by leukocytes, or their synthetic equivalents or combinations thereof.
- 25 20. A method according to any one of claims 16-19 wherein the antibody is in the form of the supernatant harvested from the culture medium.
- 30 21. An antibody against a Mycoplasma prepared according to the method of any one of claims 16-20.

22. A method of identifying a putative protective antigen associated with a Mycoplasma, preferably Mycoplasma hyopneumoniae, said method including providing

- 5 a sample of a Mycoplasma; and
 an antibody probe including at least one antibody against a Mycoplasma;
 probing the Mycoplasma sample with the antibody probe to detect at least one antigen; and
10 isolating the antigen detected.

23. A method of purifying a putative protective antigen associated with a Mycoplasma, preferably Mycoplasma hyopneumoniae, said method including providing

- 15 a crude antigen mixture; and
 an antibody against a Mycoplasma immobilized on a suitable support;
 subjecting the crude antigen mixture to affinity chromatography utilizing the immobilized antibody; and
20 isolating the purified antigen so formed.

24. A method for preparing a synthetic antigenic polypeptide against Mycoplasma, preferably Mycoplasma hyopneumoniae, which method includes providing

- 25 a cDNA library or genomic library derived from a sample of Mycoplasma; and
 an antibody probe including an antibody prepared according to claim 16;
 generating synthetic polypeptides from the cDNA library or genomic library;
30 probing the synthetic polypeptides with the antibody probe; and
 isolating the synthetic antigenic polypeptide detected thereby.

- 32 -

25. A method according to claim 24 wherein the antibody probe includes an antibody raised against an antigen against Mycoplasma hyopneumoniae, or related infections, selected from the group of antigens having approximate molecular weights of 110-114, 90-94, 72-75, 60-64, 52-54 and 46-48 kilodaltons
5 (kD), as herein described, mutants, derivatives and fragments thereof.

26. A synthetic putative protective antigen in the 72-75 kD region produced by a method according to claim 24 or 25 having an N-terminal amino acid sequence:

AGXLQKNSLLEEVWYLAL

10

27. A synthetic putative protective antigen according to claim 26 further including internal amino acid sequences:

AKNFDFAPSIQGYKKIAHEL

NLKPEQILQLLG

15

LLKAEXNKXIEEINTXLDN

28. A synthetic putative protective antigen in the 60-64 kD region produced by a method according to claim 24 or 25 having an N-terminal amino acid sequence:

MKLAKEKGFX(N/L)(M/V)IK

20

ADP(F/I)(R/E)Y(V/A)PQG(Q/A)X(M/N)VG

29. A synthetic putative protective antigen in the 52-54 kD region produced by a method according to claim 24 or 25 having an n-terminal amino acid sequence:

AGXWAKETTKEEKS

25

30. A synthetic putative protective antigen according to claim 29 further including internal amino acid sequences:

AWVTADGTVN

AIVTADGTVNDNKPNQWVRKY.

30

31. A synthetic putative protective antigen in the 46-48 kD region produced by a method according to claim 24 or 25 having an N-terminal amino acid sequence:

- 33 -

AGXGQTESGSTSDSKPQAETLKHKV

32. A synthetic putative protective antigen according to claim 31 further including internal amino acid sequences:

5 TIYKPDVKLGKVAVEVLRVIAKKNKASR
 AEQAITKLKLEGFDTQ
 KNSQNKIIDLSP EG

33. A vaccine or veterinary composition including a prophylactically effective
10 amount of at least one putative protective antigen against a Mycoplasma
according to any one of claims 1-13.

34. A vaccine or veterinary composition according to claim 33 including a plurality of putative protective antigens selected from antigens having
15 approximate molecular weights of 110-114, 90-94, 72-75, 60-64, 52-54 and 46-48 kilodaltons.

35. A vaccine or veterinary composition including an antibody against a
Mycoplasma according to claim 21.

20 36. A diagnostic kit including a diagnostic antigen or fragment thereof according to any one of claims 1-13 and 26-32.

37. A method for preventing or treating a Mycoplasma infection, which method
25 including administering to an animal a prophylactically or therapeutically effective amount of at least one putative protective antigen according to any one of claims 1-13.

38. An isolated DNA fragment encoding a putative protective antigen against
30 Mycoplasma or related infections, said DNA fragment having a nucleic acid sequence according to Figure 6 or an homologous sequence, and functionally active fragments, mutant, variant or recombinant thereof.

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39. A clone including a DNA fragment according to claim 38.
40. A clone according to claim 39 which is clone pC1-2 as hereinbefore described.
41. An amino acid sequence or functional equivalent thereof encoded by the DNA fragment according to claim 38.
- 10 42. An amino acid sequence or functional equivalent thereof having the amino acid sequence of Figure 7.
43. A putative protective antigen or antibody substantially as hereinbefore described with reference to the examples.

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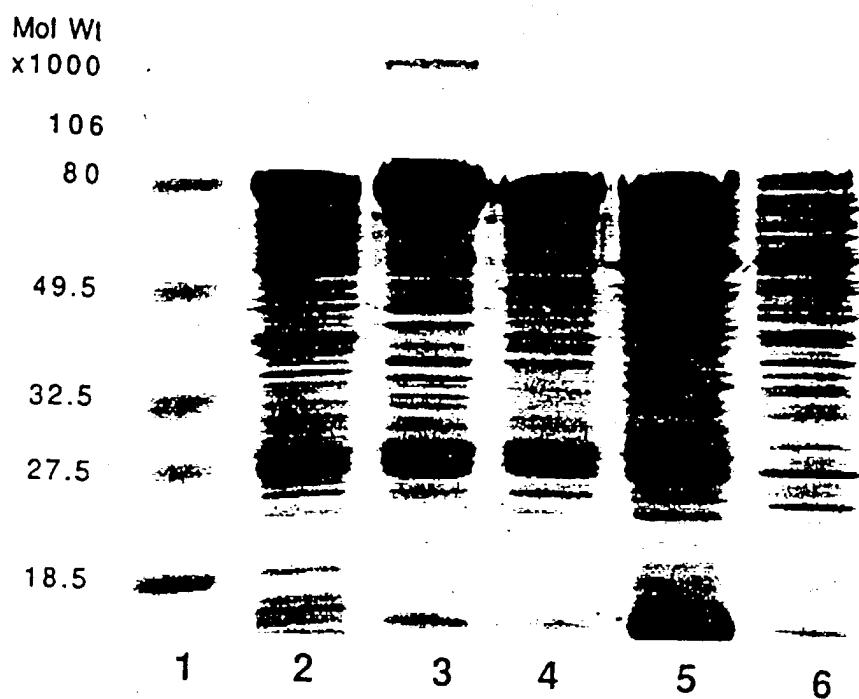


FIG. 1

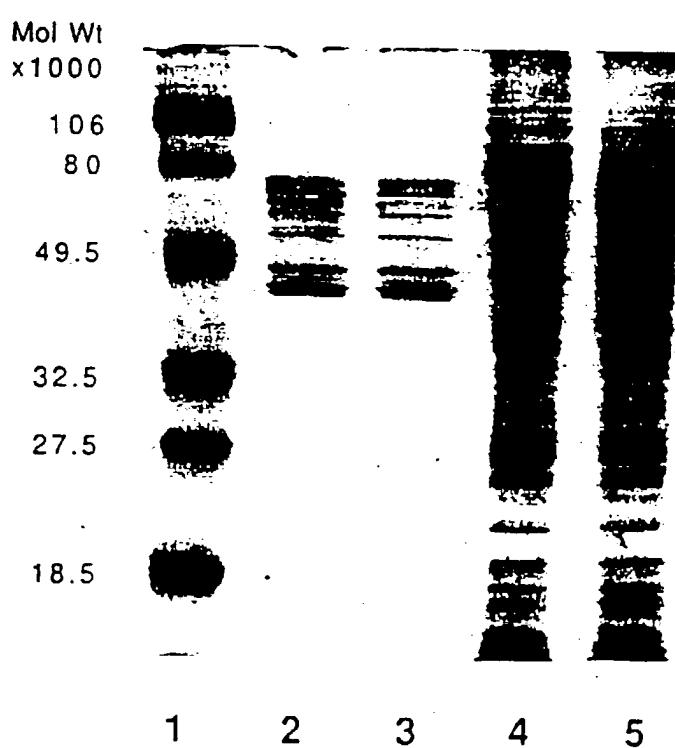


FIG. 2

2/4

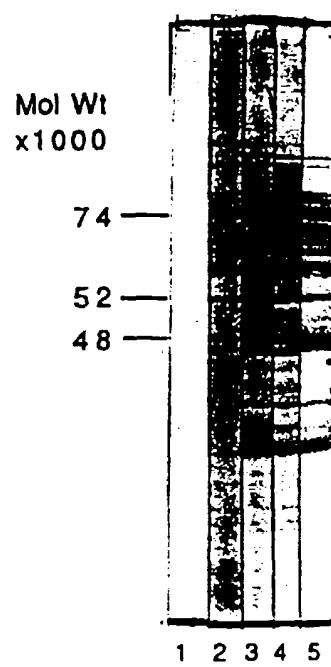


FIG. 3

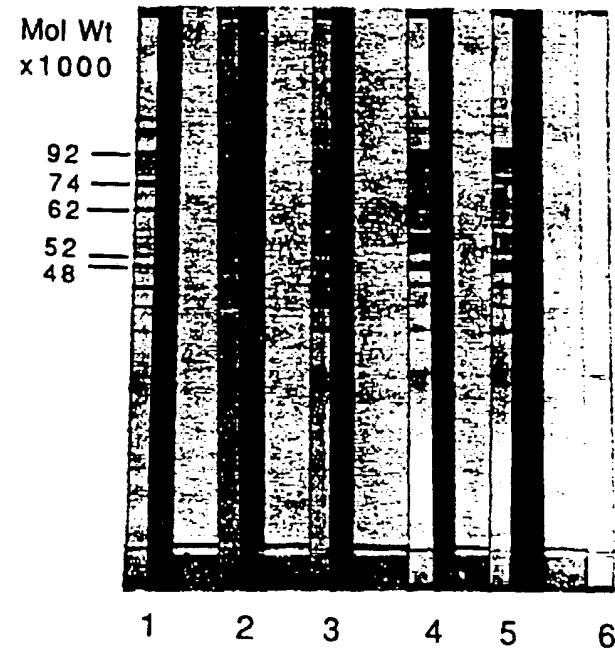


FIG. 4

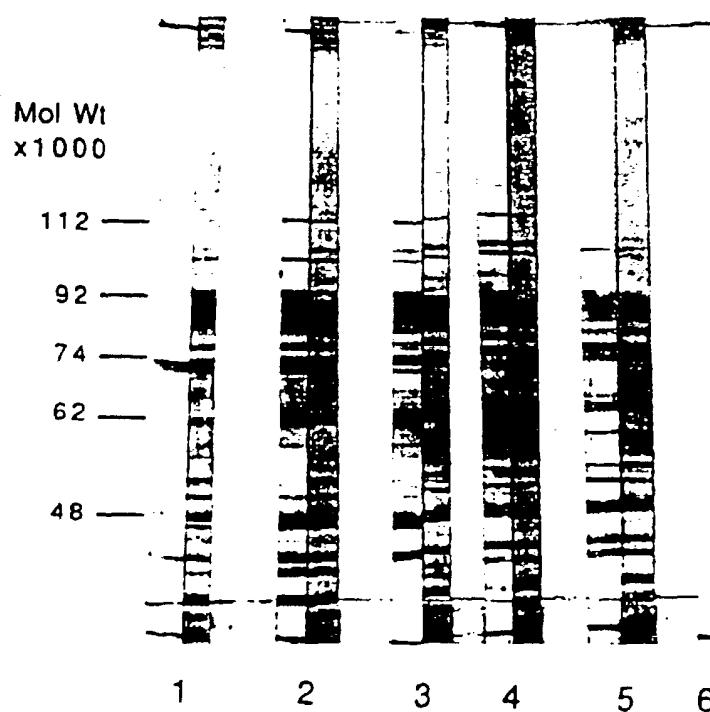


FIG. 5

10	20	30	40	50
1234567890	1234567890	1234567890	1234567890	1234567890
ATGAAAAAAA	TGCCACTATA	CCAGAGGAAA	GAGCAGTATA	TAAGATAATT
AAAATTACAT	TTTCTTCATT	TGCGCCAGAA	TTTTAAGAA	TTAGTACATT
AAAAGTAGA	ACAAAAGTTA	TTAATGTAAA	CATTAGCGCA	ATCCTTAAGA
AAAATTAAA	AGTTTTATCT	ATTTTTTTA	ATCGAAATCC	AACCAGGCAT
AAATCTTGT	CAGTATTAT	CAAGTCGGTA	TTTTTCATT	ATTTCTACTA
AAATATTATT	TGAATTGCA	TTTCCATAA	TCTAAAATT	TACATTTTT
TATAACAATT	TTTAAAAATT	ACTCTTAAT	TTATAGTATT	TTTTTATTTT
TTAGTCTAAA	TTATAAAATT	ATCTTGAATT	TTATTGAAT	TTTTATATT
TAGTACTAAA	AAATACAAAT	ATTTTTCCT	ATTCTAAGAA	AAATTCAATT
TTTAAAAAAA	ATTGATTTT	ATAGTATAAT	TTGTTGTAT	AATTGAATTA
ACTTGATTTG	AAAGGGAAACA	AAATGAAAAA	AATGCTTAGA	AAAAAATTCT
TGTATTCATC	AGCTATTAT	GCAACTTCGC	TTGCATCAAT	TATTGCATTT
GTTGCAGCAG	GTTGTGGACA	GACAGAATCA	GGTTCAACTT	CTGATTCTAA
ACCACAAAGCC	GAGACGCTAA	AACATAAAAGT	AAGTAATGAT	TCTATTGAA
TAGCACTAAC	CGATCCGGAT	AATCCTCGAT	GAATTAGTGC	CCAAAAAGAT
ATTATTTCTT	ATGTTGATGA	AACAGAGGCA	GCAACTTCAA	CAATTACAAA
AAACCAGGAT	GCACAAAATA	ACTGACTCAC	TCAGCAAGCT	AATTAAAGCC
CAGCGCCAAA	AGGATTTATT	ATTGCCCTG	AAAATGGAAG	TGGAGTTGGA
ACTGCTGTTA	ATACAATTGC	TGATAAAGGA	ATTCCGATTG	TTGCCTATGA
TCGACTAATT	ACTGGATCTG	ATAAATATGA	TTGGTATGTT	TCTTTGATA
ATGAAAAGT	TGGTGAATT	CAAGGTCTTT	CACTTGCTGC	GGGTCTATTA
GGAAAAGAAG	ATGGTGCTTT	TGATTCAATT	GATCAAATGA	ATGAATATCT
AAAATCACAT	ATGCCCAAG	AGACAATTTC	TTTTTATACA	ATCGCAGGTT
CCCAGAGATGA	TAATAATTCC	CAATATTTT	ATAATGGTGC	AATGAAAGTA
CTTAAAGAAT	TAATGAAAAA	TTCGAAAAT	AAAATAATTG	ATTTATCTCC
TGAAGGCGAA	AATGCTGTT	ATGCCCAGG	ATGAAATTAT	GGAACGCCG
GTCAAAGAAT	CCAATCTTT	CTAACAAATT	ACAAAGATCC	AGCAGGTGGT
AATAAAATCA	AAGCTGTTGG	TTCAAAACCA	GCTTCTATT	TCAAAGGATT
TCTTGCCCCA	AATGATGGAA	TGGCCGAACA	AGCAATCACC	AAATTAAAC
TTGAAGGGTT	TGATACCCAA	AAAATCTTG	TAACTCGTCA	AGATTATAAT
GATAAAGCCA	AAACTTTAT	CAAAGACGGC	GATCAAATA	TGACAATT
TAAACCTGAT	AAAGTTTAG	GAAAAGTTGC	TGTTGAAGTT	CTTCGGGTTT
TAATTGCAAA	AAAAAATAAA	GCATCTAGAT	CAGAAGTCGA	AAACGAACTA
AAAGCAAAAC	TACCAAATAT	TTCATTTAAA	TATGATAATC	AAACATATAA
AGTACAAGGT	AAAAATATTA	ATACAATT	AGTAAGTCCA	GTAATTGTTA
CAAAAGCTAA	TGTTGATAAT	CCTGATGCCT	AA	1782

10	20	30	40	50
1234567890	1234567890	1234567890	1234567890	1234567890

MKKMLRKFL YSSAIYATSL ASIIAFVAAG CGQTESGSTS DSKPQAETLK	50
HKVSNDSIRI ALTDPDNP RW ISAQKDIISY VDETEAATST ITKNQDAQNN	100
WLTQQANLSP APKGFIIAPE NGSGVGTAVN TIADKGIPIV AYDRLITGSD	150
KYDWYVSDN EKVGE LQGLS LAAGLLGKED GAFDSIDQMN EYLKSHMPQE	200
TISFYTIAGS QDDNNNSQYFY NGAMKVLKEL MKNSQNKIID LSPEGENAVY	250
VPGWNYGTAG QRIQSFLTIN KDPAGGNKIK AVGSKPASIF KGFLAPNDGM	300
AEQAITKLKL EGFDTQKIFV TRQDYNDKAK TFIKDGDQNM TIYKPDKVLG	350
KVAVEVLRVL IAKKNKA SRS EVENELKA KL PNISFKYDNQ TYKVQGKNIN	400
TILVSPVIVT KANVDNPDA	419

FIG. 7

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/AU 96/00149

A. CLASSIFICATION OF SUBJECT MATTERInt Cl⁶: C07K 16/12; C12N 15/31; C12P 21/00, 21/02, 21/08; G01N 33/53, 33/531; A61K 39/04; C07K 14/30

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC As Above

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AU: IPC As AboveElectronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT, BIOT, JAPIO: MYCOPLASM: AND ANTIGEN#
CASM: MULTI-SEQUENCES**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	AU,A, 70685/87 (CETUS CORP) 1 October 1987 Example 1, Claims	1,16,17,20-23,33 35-37,43
X	AU,B, 49035/90 (640364) (UNIVERSITY OF MELBOURNE et al) 11 October 1990 Claims	1,2,16,20,22
X	AU,A, 76820/91 (SYNERGEN, INC.) 17 October 1991 Fig. 1, 4, 6 and 7, Claim 3	14,38,39,41,42



Further documents are listed in the continuation of Box C



See patent family annex

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier document but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search
6 May 1996Date of mailing of the international search report
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INTERNATIONAL SEARCH REPORT

International Application No.

PCT/AU 96/00149

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	AU,A, 17602/95 (SYNERGEN, INC.) 26 October 1995 Fig. 1, 4, 6 and 7 P 13 L14-P14 L5	14,38,39,41,42
X	AU,B, 49599/90 (638970) (AUSPHARM INTERNATIONAL LTD.) 26 July 1990 Examples 4 and 5	16,17,20,21,35
X	US,A, 4894332 (SCHALLER et al) 16 January 1990 Examples	1,2,14,16,17, 20-22,24,33, 35,37-39,41,42
X	US,A, 5252328 (FAULDS et al) 12 October 1993 Examples 1 to 4	1-17,20-23, 33-35,37
X	US,A, 5240706 (FAULDS) 31 August 1993 Examples 1 and 2, Claims	1-6,14-16,21 22,24,25,33-35 37,38,41,42
X	EP,A, 0475185 (NIPPON FLOUR MILLS CO., LTD.) Refer Example 1, Claims	1-6,12-17, 20-23,33-35, 37-39,41,42
PX	Journal of Bacteriology, Vol. 177, No. 7, April 1995, pp1915-1917, "Molecular Cloning of a 46-Kilodalton Surface Antigen (P46) Gene from <u>Mycoplasma hyopneumoniae</u> : Direct evidence of CGG Codon Usage for Arginine", Futo et al	3-6,12-15,24 25,31,32,38, 39,41,42
PX	Journal of Clinical Microbiology, Vol. 33, No. 3, March 1995, pp 680-683, "Recombinant 46-Kilodalton Surface Antigen (P46) of <u>Mycoplasma hyopneumoniae</u> Expressed in <u>Escherichia Coli</u> Can Be Used for early Specific Diagnosis of Mycoplasmal Pneumonia of Swine by Enzyme-Linked Immunosorbent assay", Futo et al	3-6, 12-15,38,39, 41,42
X	Infection and Immunity. Vol. 49, No. 2, pp329-335, "surface Proteins of <u>Mycoplasma hyopneumoniae</u> Identified from an <u>Esherichia coli</u> Expression Plasmid Library", Klinkert et al	3-6,12-15,38 39,41,42
A	EP,A, 0571648 (WENG) 1 December 1993	
A	Derwent abstract Accession No. 88-010509, Class 503, JP,A, 62-273455 (NORIINSHO KK) 27 November 1987	
A	Derwent Abstract Accession No. 90-241949, Class 503, JP,A, 02-167079 (NIPPON SEIFUN KK) 27 June 1990	
A	Derwent abstract Accession No. 95-203749, Class B04, C06, D16, JP,A, 07-118167 (ZENKOKU NOGYO KYODO KUMIAI RENGOKAI) 9 May 1995	

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/AU 96/00149

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

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		FI	900498	IL	93234	JP	3087199
		NO	178893	NZ	232279	ZA	9000766
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		FI	924428	HU	65827	JP	5506984
		NO	923828	WO	9115593	US	5459048
		AT	135048	AU	622855	DE	3751727
		DK	1608/88	EP	315637	HU	208550
		IL	83324	JP	1503735	WO	8800977
AU	95/17602	AU	76820/91	CA	2078131	EP	527771
		FI	924428	HU	65827	JP	5506984
		NO	923828	WO	9115593	US	5459048
		AT	135048	AU	622855	DE	3751727
		DK	1608/88	EP	315637	HU	208550
		IL	83324	JP	1503753	WO	8800977
AU	90/49599	EP	454735	NZ	232190	WO	9007935
		ZA	9000474				
US	4894332	CA	1301677	CN	86102858	EP	196215
		JP	61274687				
/							
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INTERNATIONAL SEARCH REPORT

International Application No.

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Patent Document Cited in Search Report				Patent Family Member			
US	5252328	CA	1321142	CN	88101554	DK	1674/88
		EP	283840	HU	203672	IE	61626
		JP	63258427	PT	87041		
US	5240706	AT	134705	DE	68925769	EP	359919
		JP	2291271				
EP	475185	JP	5091882				
END OF ANNEX							